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ANNOTATIONS, MINOR CONTRIBUTIONS, QUERIES

Under the above caption will be published from time to time comments criticisms and suggestions on technical procedures; minor contributions such as laboratory aids and short cuts which are not considered sufficiently important to warrant a formal paper; and queries.

Obviously comments and criticisms should be signed; queries should be signed but names will be withheld on request; full credit will be given those who contribute laboratory aids, short cuts and the like.

An attempt will be made to obtain answers from authoritative sources to the queries submitted. It must be emphasized that the views expressed in this department are not the opinions of any official body.

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TECHNIC OF THE PROTHROMBIN TIME DETERMINATION*

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Quick has published a number of papers in which he has described the technic of performing the prothrombin time determination. As I have had a considerable experience with this test, it was thought worth while to indicate briefly the exact procedure which has been found to be quite satisfactory.

Blood is collected in a round-bottomed test tube in which 0.5 cc. of tenth-molar solution of sodium oxalate has been placed. Four and a half centimeters of blood are added and, placing the finger over the end of the tube, it is inverted four or five times to assure mixing. The blood is allowed to sediment half an hour or is centrifuged for three minutes at low speed. Place 0.1 cc. of the plasma in a tube, 13 by 100 mm., and add 0.1 cc. of thromboplastin, mixing gently so as to avoid bubbles. The mixture is now warmed in a water bath which is kept at 37.5°C. The temperature of the bath is important and should not vary over one degree. Keeping the tube in the bath, quickly introduce 0.1 cc. of fortieth-molar calcium chloride and agitate the tube quickly. At the instant when the calcium chloride is added, a stop-watch or clock should be started and the exact time recorded for the formation of a firm clot (semi-solid). In order to determine the second at which the clot is developed, the tube should be tipped every few seconds in order to determine when the clot has formed.

Because of the importance of keeping the water bath at an accurate temperature figure 1 is reproduced to illustrate a satisfactory, inexpensive bath. It is made by using a square 6-inch museum jar, approximately 9 inches deep, or a round jar of 6 inch diameter. The immersion knife type heater is 125 watts and has a neon bulb inserted in the top to indicate when the current is on. The temperature control is a DeKhotinsky bimetallic, single pole, single throw thermoregulator, and is used with a 0.1 mfd. condenser. In order to keep the bath stirred a small jet of compressed air is kept bubbling through close to the knife heater.

Thromboplastin is prepared from rabbit brain by the following method: A

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rabbit is killed by injecting 20 cc. of air into the lateral vein of the ear. The skull is quickly opened and the brain removed. It is freed of meninges and blood and then triturated with a spatula on a glass plate. After it has been intimately triturated it is spread thin on the plate and allowed to dry in the incubator for forty-eight hours. The dried brain is then scraped up and kept in the icebox in tightly stoppered bottles. Usually the dried brain will keep at least two months.

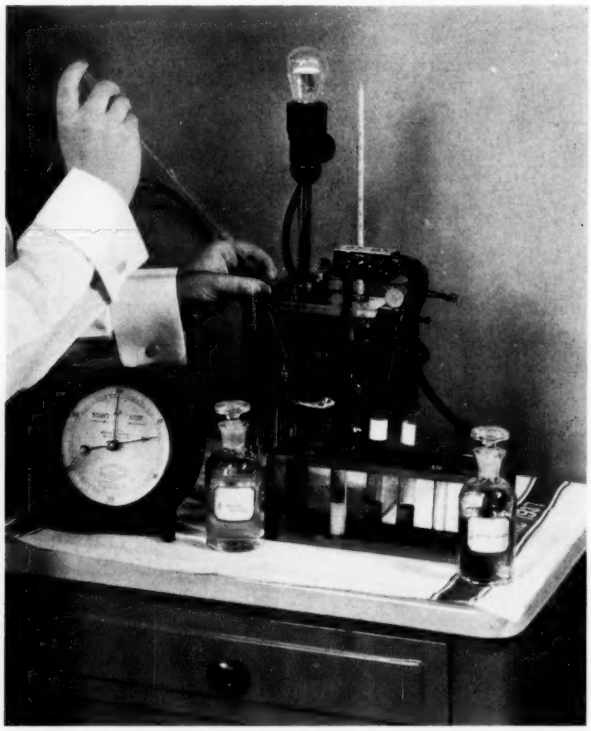

FIG. 1. SIMPLE, ACCURATE WATER BATH, REAGENTS AND APPARATUS NECESSARY TO PERFORM PROTHROMBIN TESTS

For the test, 0.5 gm. of this dried brain material is mixed with 5 cc. of physiologic salt solution. This is incubated at 56°C. for fifteen minutes and centrifuged at low speed for approximately five minutes. The supernatant liquid is used as "thromboplastin;" it should be used within a week. The tenth-molar sodium oxalate is made by dissolving 1.34 gm. of sodium oxalate in 100 cc. of distilled water and the fortieth-molar calcium chloride by dissolving 0.28 gm. of anhydrous calcium chloride in 100 cc. of distilled water.

It is obvious from this description that the preparation of the materials and the test should not be attempted by any but the more skilled in biologic reactions, since the test is delicately balanced and the procedure must be precise and carefully executed. By this method normal human plasma clots in eighteen to twenty-two seconds and with each preparation of thromboplastin a known normal or series of normals should be run. If a specimen gives a clotting time of more than thirty seconds, make a second determination on the same specimen in order to check the first result. It is best to report the test with the average normal reading. The necessity for accurate timing can be appreciated by realizing that a clotting time of thirty seconds is abnormally high when the normal is twenty seconds. A determination of the error in performing the test has been made and it has been found that when the clot forms in twenty seconds there is a probable error of ± 0.6 second; while when the clotting time is fifty seconds the probable error is ± 2 seconds. From the work of Quick and from my determinations it seems likely that when the prothrombin time is as long as thirty seconds the blood contains about 25 per cent of normal prothrombin. By testing serial dilutions of serum it is possible to arrive at a rough approximation of the relative amount of prothrombin present, but further investigation is necessary before a reliable technic of this type can be described.

A NEW FROZEN SECTION METHOD

For the Preparation of Permanent Frozen Sections of Loose Texture Tissues*

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The much neglected frozen section method could advantageously be employed in routine section cutting for its rapidity,

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economy, preservation of normal structure of cell components and adaptability to many special staining methods.

A principle obstacle to the preparation of routine permanent sections by the freezing method has been the difficulty in keeping intact the loose textured or necrotic tissues, such as abscessed lungs, degenerated tumor masses, congested spleen, kidney glomeruli, endometrial tissue, testicle, thymus, etc.

Recently I have overcome this defect by developing an effective method of infiltrating such tissues with blood serum and then coagulating with dioxane. This permits the preparation of complete frozen sections from biopsy and autopsy material, thus avoiding the delay and extra unnecessary expense of preparing routine paraffin sections.

THE METHOD ("SERUMIZING")

The loose texture tissues are first fixed in 10 per cent commercial formaldehyde in the usual manner for 24 hours or longer. Then the blocks are trimmed and washed several times in tap water to remove the formaldehyde, and placed in a small stender dish or specimen bottle containing uncontaminated human or animal serum (fresh sera from Wassermann test is ideal). The amount of the serum should be ample to cover all the tissues, and the dish or the bottle (tightly covered) put in a warm place overnight (the top of paraffin oven is a good place for it). Any floating tissue such as lung or fatty material should be covered with absorbent cotton in order to keep all parts in the serum.

Decant the excess serum after overnight infiltration, and without washing add sufficient dioxane to cover the blocks and let stand for 3 to 5 hours or until complete coagulation occurs. Then without further fixation, tissue blocks are frozen on a freezing microtome with CO_2 gas, and sections are cut, stained and mounted in usual manner.

The blocks can not be kept in dioxane indefinitely, because it causes slow shrinkage to the tissue. After section cutting, place them in 10 per cent formaldehyde for permanent keep.

For emergency examination very thin blocks of formaldehyde-fixed tissue are placed in serum in a paraffin oven at 56°C. for 1 to 2 hours, then coagulated in dioxane for one hour, when they will be ready for sectioning.

This method causes very little shrinkage, but no curling or shriveling of sections as is expected in other embedding processes intended for frozen sections, such as gelatin embedding methods described in microtechnics.

The infiltrating serum, after it is completely coagulated by the action of dioxane, acts as a supporting medium to furnish stability and to hold cells and

intracellular structure in proper relation to each other, and does not interfere with any staining process as is the case in gelatin method.

FIG. 1

FIG. 2

FIGS. 1 AND 2. METASTATIC CARCINOMA OF LIVER

Sections with ragged edges and holes are prepared with regular frozen technic.

The same blocks treated with method described yield complete sections.

This serumizing process makes hard tissues, such as bone and uterus, soft and pliable, thus producing better sections.

For convenience, fresh serum is stored in small bottles and preserved with the addition of 2 cc. of commercial (40 per cent) formaldehyde to 50 cc. of the fluid, which keeps well at room temperature for several weeks without contamination.

PROCEDURES RECOMMENDED FOR TRIAL

PHOTELOMETRIC (SHEARD-SANFORD) DETERMINATIONS OF BLOOD CYANATE, BLOOD SULFAPYRIDINE, URINE SUGAR AND TOTAL PROTEINS IN SPINAL FLUID

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The universal adaptability of the Sheard-Sanford Photelometer to all types of chemical analyses in the clinical laboratory no longer requires comment. With this apparatus it is possible to perform accurately quantitative estimation of chemical metabolites in blood, urine and other body fluids where heretofore the titration method, colorimetry or nephelometry has been in use.

The colorimetric determinations of blood cyanate, blood sulfapyridine and urine sugar and nephelometric estimation of total proteins in spinal fluid, by Griffith and Lindauer[1], Marshall and Litchfield[2], Sumner[3], and Ayer, Dailey and Smith[4], respectively, have recently been adapted by us to the use of the Sheard-Sanford Photelometer, with eminently satisfactory and accurate results.

We are, therefore, presenting these four procedures for further trial and evaluation.

PHOTELOMETRIC DETERMINATION OF BLOOD CYANATE

(Adapted from Method by Griffith and Lindauer, Colorimetric)[1]

Procedure

1. In a small Erlenmeyer flask place 7 cc. 10 per cent trichloracetic acid and 7 cc. serum (add slowly with shaking).
2. Let stand 10–15 minutes.
3. Filter through a small retentive filter paper. The filtrate should be perfectly clear; if it is not, filter again through the same filter paper.
4. In a small absorption cell (6 cc.) of the photelometer place 5 cc. filtrate and 1 cc. ferric nitrate reagent.
5. Mix and read in the Photelometer.

Reagents

1. Ferric nitrate reagent: 50 grams crystalline ferric nitrate, 500 cc. distilled H_2O and 25 cc. concentrated nitric acid. Make up to 1000 cc. with distilled H_2O.

2. 10 per cent trichloracetic acid: 10 grams trichloracetic acid and 100 cc. distilled H_2O.

3. Standard stock solution: 1 gram potassium thiocyanate and 800 cc. distilled H_2O. Titrate 20 cc. silver nitrate (made by dissolving exactly 2.9185 grams silver nitrate in 1 liter distilled H_2O) acidified with 5 cc. concentrated nitric acid with the potassium thiocyanate solution using ferric ammonium sulphate as an indicator. Calculate dilution so that 20 cc. thiocyanate is equivalent to 20 cc. silver nitrate solution. Dilute and titrate again.

Technique of Calibrating Photelometer (Sanford-Sheard)

1. Make following dilutions of stock standards: 2 cc. stock standard diluted to 100 cc. with distilled H_2O, 0.1 mg. standard; 4 cc. stock standard diluted to 100 cc. with distilled H_2O, 0.2 mg. standard; 7 cc. stock standard diluted to 100 cc. with distilled H_2O, 0.35 mg. standard; 10 cc. stock standard diluted to 100 cc. with distilled H_2O, 0.5 mg. standard; 15 cc. stock standard diluted to 100 cc. with distilled H_2O, 1.5 mg. standard.

2. In large test tubes place 5 cc. of each of above dilutions, 5 cc. 10 per cent trichloracetic acid and 2 cc. ferric nitrate reagent.

3. Mix and read on photelometer using orange filter: 0.1 mg. standard is equivalent to 2 mg. blood cyanate; 0.2 mg. standard is equivalent to 4 mg. blood cyanate; 0.35 mg. standard is equivalent to 7 mg. blood cyanate; 0.5 mg. standard is equivalent to 10 mg. blood cyanate; 1.5 mg. standard is equivalent to 15 mg. blood cyanate. Plot results on semi logarithmic paper.

PHOTELOMETRIC DETERMINATION OF SULFAPYRIDINE IN BLOOD

(Adapted from Method of Marshall, Colorimetric)[2]

Procedure

A. *Total Sulfapyridine:*
   a. Place 10 cc. filtrate (*vide infra*) in a test tube.
   b. Add 1 cc. 2 N HCl.
   c. Heat in boiling water bath one hour.
   d. Cool and make up to 10 cc. with distilled H_2O.
   e. Transfer this quantitatively to a 50 cc. Erlenmeyer flask. From this point the same procedure is followed for both total and free sulfapyridine.

B. *Free Sulfapyridine:*
   a. In a 50 cc. Erlenmeyer flask place 10 cc. filtrate 1.
   b. To both total and free flasks add 1 cc. sodium nitrite. Let stand three minutes.
   c. To free sulfapyridine add 1 cc. 1 molar phosphate buffer (containing 0.5 per cent ammonium sulfamate). Total sulfapyridine add 1 cc. 2 molar phosphate buffer. Let stand two minutes.
   d. Add 5 cc. dimethyl-alpha-naphtholamine. Let stand ten minutes.
   e. Read in Photelometer.

Filtrate

1. In an Erlenmeyer flask place 4 cc. oxalated blood and 28 cc. saponin solution. Let stand about 2 minutes for complete laking.
2. Add 8 cc. 15 per cent trichloracetic acid with continuous shaking.
3. Let stand five minutes. Filter using ashless filter paper.

Reagents

1. Trichloracetic acid 15 per cent: 15 grams trichloracetic acid. Dilute to 100 cc.
2. 0.1 per cent sodium nitrite: 1 gram sodium nitrite and 1000 cc. distilled H_2O.
3. Saponin solution: 0.5 gram saponin per liter distilled H_2O.
4. Dimethyl-alpha-naphthalamine: 1 cc. stock dimethyl-alpha-naphthalamine and 100 cc. 95 per cent alcohol.
5. 1 molar phosphate buffer: 13.8 grams sodium dihydrogen phosphate; 0.5 gram ammonium sulfamate and 100 cc. distilled H_2O.
6. 2 molar phosphate buffer: 27.6 grams sodium dihydrogen phosphate; 0.5 gram ammonium sulfamate and 100 cc. distilled H_2O.
7. Stock sulfapyridine: 200 mg. sulfapyridine. About 700 cc. warm (50°) distilled H_2O. Shake vigorously. Make to volume.

Technique for Calibrating Photelometer (Sanford-Sheard)

1. From stock sulfapyridine standard make up the following standards: 1 cc. standard stock plus 18 cc. trichloracetic acid diluted to 100 cc. with distilled H_2O equal 0.2 mg. standard (10 cc., 2 mg.); 2.5 cc. standard stock plus 18 cc. trichloracetic acid diluted to 100 cc. with distilled H_2O equal 0.5 mg. standard (10 cc., 5 mg.); 5 cc. standard stock plus 18 cc. trichloracetic acid diluted to 100 cc. with distilled H_2O equal 1 mg. standard (10 cc., 10 mg.); 6 cc. standard stock plus 18 cc. trichloracetic acid diluted to 100 cc. with distilled H_2O equal 1.2 mg. standard (10 cc., 12 mg.); 7 cc. standard stock plus 18 cc. trichloracetic acid diluted to 100 cc. with distilled H_2O equal 1.4 mg. standard (10 cc., 14 mg.).
2. Place 10 cc. of each of the above standards in a 50 cc. Erlenmeyer flask and treat the same as unknown.
3. Read on photelometer using a blue filter and plot results on semi-logarithmic paper.

PHOTELOMETRIC DETERMINATION OF SUGAR IN URINE

(Adapted from Method of Sumner, Colorimetric)[3]

Procedure

1. For urines having a trace or one plus sugar (qualitative test) do not dilute. For two plus to four plus dilute urine 1:10.

2. Place 1 cc. urine or diluted urine in a test tube graduated at 25 cc.
3. Add 3 cc. dinitrosalicylic acid reagent.
4. Place in boiling water bath five minutes.
5. Cool in runing water and dilute to the mark.
6. Read in Photelometer.

Reagents

Dinitrosalicylic Acid Reagent:

1. Dissolve 200 grams sodium potassium tartrate in about 1000 cc. warm distilled water.
2. Dissolve 24 grams sodium hydroxide in about 200 cc. distilled water and add to "1". Let cool.
3. Add to this mixture 6 grams crystalline phenol and 6 grams sodium bisulphite.
4. Dissolve 14 grams dinitrosalicylic acid (Eastman) in about 200 cc. of boiling distilled water when dissolved let cool somewhat and add to the previous solution and make up to 2000 cc. with distilled H_2O.

Technique of Calibration of Photelometer (Sanford-Sheard)

1. Make up glucose standards from 0.05 to 1 per cent (suggested ones being 0.05, 0.1, 0.3, 0.5, 0.7 and 1 per cent.
2. Treat these standards as the unknown.
3. Make the photelometric readings using a wratten filter No. 6 (yellow) and plot on semi-logarithmic paper.

PHOTELOMETRIC ESTIMATION OF TOTAL PROTEINS IN SPINAL FLUID

(Adapted from Methods of Dennis and Ayer, Nephelometric)[4]

Procedure

1. In a test tube place 3 cc. spinal fluid; 2 cc. distilled H_2O and 5 cc. sulfosalicylic acid (5 per cent solution).
2. Let stand 5 minutes.
3. Read in Photelometer.

Technique

1. Obtain about 30 cc. of fresh normal blood serum.
2. Dilute 25 cc. of this serum to 250 cc. in a volumetric flask with 15 per cent sodium chloride. Filter. This is the concentrated stock standard.
3. Determine the total nitrogen by Kjeldahl Method.
4. Determine the non-protein nitrogen of the original undiluted serum.
5. Subtract one-tenth of the serum non-protein nitrogen figure from the total nitrogen content of the concentrated stock standard and multiply the result by 6.25 to get the protein content of the stock standard.

6. From this concentrated stock standard make dilution from 10 to 80 mgm. of protein using the following formula.

7. Milligrams desired times cubic centimeters of dilute stock standard desired over protein concentration in milligrams per 100 cc. of concentrated stock standard equals cubic centimeters of concentrated stock standard required.

8. Treat these dilute standard solutions of protein the same as the unknown.

9. Head in photelometer using a clear filter and plotting the results on semi-logarithmic paper. This results in a linear line from which the concentrations of the unknown solution may then be read.

NOTES

If the protein concentration is greater than 80 mg. greater dilutions of the spinal fluid should be made.

Spinal fluids which are contaminated with macroscopic blood or bacteria are unsatisfactory for protein determinations.

Fluids which are allowed to stand even though they are corked will give increasing amounts of protein from day to day.

REFERENCES

(1) GRIFFITH, JOHN Q. AND LINDAUER, M. A.: Am. Heart J., **14:** 710, 1937.

(2) MARSHALL, E. K., JR. AND LITCHFIELD, J. T., JR.: Science, **88:** 85 (July 22), 1938.

(3) SUMNER, J. B.: J. Biol. Chem., **65:** 393, 1925.

(4) AYER, JAMES, DAILEY, MARY ELIZABETH AND SMITH, FRANK: Arch. of Neur. and Psych., **26:** 1038, Nov., 1931.